Multiple Primary Melanoma Revisited

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BACKGROUND. Incidence of cutaneous melanoma continues to increase in the Caucasian population worldwide. Approximately 5% of melanoma patients develop additional primary melanoma. This rate is significantly higher than the estimated lifetime risk of an individual for developing the disease (1.4%). These features suggest that a genetic predisposition may underlie multiple primary melanomas (MPMs). Prior studies had identified *CDKN2A* mutations in a few MPM individuals. The objectives of this study were to determine the frequency of family history of melanoma in MPM cases, to characterize other clinical features including history of other cancer, and to determine the association with functional *CDKN2A* mutations.

METHODS. This study used a case series design. All living patients who had been seen in the Pigmented Lesion Clinic at the University of Pennsylvania and who had more than one primary invasive malignant melanoma or an invasive primary followed by an in situ melanoma were eligible for participation.

RESULTS. Individuals with MPM frequently had a family history of melanoma, dysplastic nevi (DN), and/or another cancer including basal cell carcinoma (BCC), and squamous cell carcinoma breast cancer, and a personal history of DN, and basal cell carcinoma. Germline mutations in *CDKN2A* gene were identified in 8 of 96 MPM cases (8.3%, 95% confidence interval, 6.7–9.9%).

CONCLUSIONS. These data indicate that the presence of MPM is associated with a modest incidence of a family history of melanoma, DN, or BCC and a small association with *CDKN2A* mutations. Therefore, in addition to the MPM index case, other family members can benefit from screening and regular surveillance for melanoma, DN, and BCC. ***Cancer* 2002;94:2248–55.**

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The incidence of melanoma continues to increase in the Caucasian population worldwide. Incidence of the disease in the United States has been doubling each decade. It is estimated that 1 of 70 Americans will develop melanoma during his/her lifetime.[1] Approximately 5% of melanoma patients develop additional primary melanomas, a frequency higher than that of developing a first primary melanoma in the general population (1.4%).[1–6] The occurrence of more than one primary cancer of any type suggests a field defect resulting from either an underlying genetic predisposition or a common environmental exposure.

It is known that melanoma kindreds often include members with multiple primary melanomas (MPMs).[3,7,8] Here, we asked the converse question: What fraction of individuals with MPM has a family history of melanoma and an identifiable shared genetic predisposition? To assess the association of MPM with familial melanoma and

other cancer, we performed full pedigree analysis of all mutation positive and negative individuals. The *CDKN2A* gene encodes a cyclin dependent kinase inhibitor and mutations in this gene have been identified in a subset of familial multiple melanoma kindreds as well as many melanoma cell lines.[9,10] In addition, there are other reports estimating the prevalence of *CDKN2A* mutations between 8% and 15% in individuals with MPM.[5,6,11,12] Here, we report the frequency of germline mutations in the *CDKN2A* gene among individuals with MPM identified at the Pigmented Lesion Clinic (PLC) of the Hospital of University of Philadelphia.

MATERIALS AND METHODS

Study Design

The institutional review board approved the protocol and the informed consent form. The study used a consecutive case series design. All living patients who had been examined in the PLC at the University of Pennsylvania, and who had more than one primary invasive melanoma or an invasive primary followed by an in situ melanoma were eligible for participation. Dysplastic nevi (DN) are atypical moles that are risk markers and nonobligate precursors of melanoma.[13–16] Obligatory criteria for the diagnosis of clinically DN were a dimension of 5 mm or greater and flatness (entirely flat or having a flat component). At least two of the following were also necessary: variable pigmentation; irregular, asymmetric outline; and indistinct borders.[16] Expert pathologists confirmed all diagnoses of melanoma. Only 3 of 96 individuals had their initial melanoma followed up by 1 or more in situ melanomas. Eligible patients were informed by mail about the study, and that they would be contacted by telephone. Each patient then was contacted by telephone to obtain preliminary consent for study participation. Appointments to recontact patients were made at this time. Patients who agreed to participate were sent a detailed family history questionnaire and an informed consent form. Patients were instructed on how to obtain cancer histories from their family members. After return of the signed informed consent form and the completed questionnaires, either a genetic counselor or a nurse who has been trained in cancer genetics interviewed each subject. Interviews focused on the family histories and sought to identify all family members, both affected and unaffected with melanoma, and to identify all members diagnosed with DN or cancer, as well as the ages of cancer diagnosis. All medical charts were abstracted for details of physical examination and the pathology reports. Ninety-six patients agreed to participate by giving consent and arrangements were made to have a 10–15-mL blood sample drawn.

Mutation Analysis of *CDKN2A* Gene

Isolation of DNA from blood samples

Genomic DNA was isolated from 5 mL of blood using a commercial DNA isolation kit (Puregene, Gentra Systems, MN). The DNA was dissolved in 500 μL of 10 mM Tris-HCl, pH 8.0, 1 mM ethylenediamine tetraacetic acid. Approximately 80–100 μg of DNA was obtained. For polymerase chain reaction (PCR), the DNA was diluted to 50 ng/μL, and 2 μL was used in a 50-μL PCR.

Direct Mutation Analysis

The *CDKN2A* gene is made up of three exons.[1,2] The sequences of the exons and the exon–intron boundaries are available from the Genbank (accession numbers: U12818, exon 1; U12819, exon 2; U12820, exon 3). Primers for each one of the exons were designed by using the MacVector program (Accelrys, San Diego, CA). The primer sequences are as follows: exon 1 (217 base pairs [bp]), p16.1F: 5′- GCAGCATGGAGCCTTCGGCTGAC-3′; p16.1B: 5′-GCGCTACCT GATTCCAATTC-3′; exon 2 (426 bp), p16.2F: 5′-TTCCTTTCCGTCATGCCG-3′; p16.2B: 5′-TTCTCAGATCATCAGTCCTC-3′; exon 3 (380 bp) p16.3F: 5′- GAATTCTGTTCCACACATCTTTG-3′; p16.3B: 5′-AAAACTACGAAAGCGGG-3′.

Polymerase chain reaction products were generated using an automated thermocycler (PE 9600) and commercial PCR kits. The PCR buffer was optimized using a kit from Stratagene (La Jolla, CA). We initially checked PCR products on an agarose gel for presence of the right size amplicons and then subjected them to dye terminator cycle sequencing (ABI, Foster City, CA) using one of the PCR primers and run on an ABI 377 sequencer.

Statistical Methods

After construction of a complete pedigree, family histories were analyzed to identify the side of the family most strongly effected with melanoma and other cancers. Thereafter, the number of individuals affected with 1) melanoma; 2) basal cell carcinomas (BCCs); 3) squamous cell carcinomas; 4) other cancers; and 5) DN was recorded, as was the presence or absence of *CDKN2A* mutations. Frequency distributions were used to describe the distribution of the personal and family history characteristics within this MPM case series. Chi-square analyses (Fisher exact test) were used to compare these factors between *CDKN2A* mutation positive and mutation negative individuals, and between DN positive and DN negative subjects. Analysis of variance was used to compare the ages of onset

TABLE 1
Family History of Melanoma, Dysplastic Nevi, and Other Cancer

Family history of melanoma	Family history of dysplastic nevi[a]	Other cancer in family[a]
10/96 (10%) with 3 or more affected relatives	11/44 (25%)	BCC, 31/77 (40%)
27/96 (28%) with 1 or 2 affected relatives		Breast, 26/81 (32%)
49/96 (51%) sporadic		Squamous cell carcinoma, 13/77 (17%)
7/96 (7%) with a third-degree relative		Pancreatic, 1/96 (1%)
3/96 (3%) with unknown family history		

BCC: basal cell carcinoma.
[a] The difference in the denominators reflects the number of individuals with the relevant information.

of the first melanoma, and the number of years between diagnoses between mutation positive and mutation negative individuals, and between DN and BCC positive and negative individuals.

RESULTS

Family History of Melanoma

During the period of this study (June 1, 1998 to February 5, 2001), 5540 cases of melanoma were examined at PLC, and 441 of those were diagnosed with MPM (8%). Of these individuals, 308 are alive currently and 184 are followed up in PLC. Of them, 96 individuals with MPM agreed to participate (52%; Table 1). There were 51 females and 45 male participants in this group. The rate of participation in the study was not determined by gender of the participant as the distribution was exactly similar among the nonparticipants (data not shown). The average age of the participants was 47 years with the earliest age of diagnosis at 13 years and latest age of diagnosis at 87 years. The distribution was similar for the nonparticipants.

Analysis of pedigrees indicated that 10 individuals had a family history with 3 or more relatives (first- or second-degree) affected with melanoma (10%; 95% confidence interval [CI], 5–16%) and 27 individuals had at least 1 or 2 additional family member affected with melanoma (28%; 95% CI, 20–37%). There were 7 individuals with melanoma in a third-degree relative (7%; 95% CI, 5.7–8.3%). In contrast, 49 (51%; 95% CI, 52–70%) probands were the only members in the family affected with melanoma. There were three individuals for whom no family history information was available.

Family History of DN

Dysplastic nevi diagnosed by clinical examination were present in 25% of the families of those with MPM who had at least 1 family member examined (Table 1).

TABLE 2
Personal History of BCC, DN, and Other Cancer and Age of Onset of First Melanoma

Personal history of BCC (%)	Personal history of DN (%)	Personal history of other cancer (females) (%)	Synchronous melanoma (%)	Median age of first melanoma (yrs)
38/96 (40)	39/96 (40)	Breast carcinoma, 4/51 (7)	12/96 (13)	47

BCC: basal cell carcinoma; DN: dysplastic nevi.

TABLE 3
Variation in Age of Onset of First Melanoma

Carrier status for melanoma precursor lesions	Dysplastic nevi present	Dysplastic nevi absent	Basal cell carcinoma present	Basal cell carcinoma absent
Mean age of onset of first melanoma (± SD)	41.46 (12.98)	50.40 (13.82)	51.44 (12.10)	39.8 (13.12)
Mean of present age (± SD)	55.33 (11.43)	58.08 (13.15)	61.5 (10.66)	50.6 (12.4)

SD: standard deviation.

Family History of Other Cancer

A significant fraction (40%) of these individuals reported a family history of BCC (50% in males; 95% CI, 32–50%) to 28% in females (95% CI, 22–34%; Table 1). The second most common carcinoma that was observed among the MPM individuals or other family members was breast carcinoma (32%; 95% CI, 32–37%), followed by squamous cell carcinoma (17%; 95% CI, 10–24%). Previous studies had documented an increased incidence of pancreatic carcinoma in *CDKN2A* mutation positive families.[17–19] We observed only one case of pancreatic carcinoma, and that was in the family of a *CDKN2A* mutation negative individual.

Personal History of Predisposing Risk Factors and Associated Malignancies

When the clinical features of individuals with MPM were analyzed, we observed that 67% of these individuals had a previous history of DN, 40% had a previous history of BCC. Of interest, among the 51 female participants, prior incidence of breast carcinoma was present among 4 (7%) of them (Table 2).

TABLE 4
Results of Mutation Analysis of *CDKN2A* ($p16^{INK4a}$) Gene

Family no.	Ethnicity	Exon	Nucleotide change	Amino acid change	Age of onset of first melanoma (yrs)	No. of melanoma	Family history
M-002	German, English, Irish	2	$ATG_{199}\rightarrow ATC_{199}$	M53I	31	4	Father with SCM
M-011	English, German	2	$G_{341}GG\rightarrow T_{341}GG$	G101W	64	2	Father with BCC
M-041	German	2	279del14	Frameshift, Stop at AA114[c]	36	3	Multiple members with SCM
M-063	Unknown	1	23Ins24bp	Duplication amino acids 11 through 18	13	7	Mother with MPM
M-082	German, English, Irish	2	$G_{341}GG\rightarrow T_{341}GG$	G101W	21	6	Paternal grandmother with SCM
M-085	Unknown	2	$ATG_{199}\rightarrow ATC_{199}$	M53I	48	2	Father, brother with SCM
M-086	Italian	2	349del16	Frameshift, stop at AA140[a]	46	2	Mother with breast carcinoma
M-064	Scottish, English, German	1	GGT→TGT	G23C[a]	32	2	Maternal grandmother with SCM
M-054	Polish	1	$GT_{122}G\rightarrow GG_{122}G$	V28G*	20	2	None

SCM: single cutaneous melanoma; BCC: basal cell carcinoma; MPM: multiple primary melanoma.
[a] Novel mutation.

Multiplicity of Melanoma and Age of Onset of First Melanoma

Among the MPM patients, 1 individual had 9 primary cutaneous melanoma primaries; another individual had 7, 2 had 6, 2 had 5, 4 had 4, 14 had 3, and the remaining 72 had 2 primaries. Approximately 13% of MPM cases presented with synchronous primary melanoma (Table 2), and 14% cases had the diagnosis of the second primary within 1 year (total 27 of 96; 28%). In a previous study, approximately 36% of individuals had synchronous melanoma.[20] The time between the diagnosis of 2 consecutive primaries for all remaining patients ranged from more than 1 year to 10 years.

The age of onset of the first melanoma ranged from 13 to 77 years with a median of 47 years. The distribution of the ages of onset of the first melanoma in the DN positive or negative individuals is given in Table 3. The mean age of onset of first melanoma is almost 10 years earlier in the DN positive group when compared with the DN negative group ($P < 0.0016$). The mean age of onset of the first melanoma for individuals with or without BCC appears different when compared without attention to the distribution of the current ages of the individuals with BCC (Table 3). This difference disappears when the current age distributions are adjusted to be the same. This result is expected because the incidence of BCC increases with age.

Spectrum of Mutations in *CDKN2A* Gene

As indicated in Table 4, we observed eight germline mutations in *CDKN2A* gene in the current cohort of patients. The missense mutations, M53I[19,21] and G101W,[22,23] and the 8–amino acid duplication starting at codon 23 have been reported before in other melanoma kindreds from all over the world.[21] The missense mutations, G23C and V28G, and the frameshift mutations, 279del14 and 349del16, translating to premature stop codons at amino acids 114 and 140, respectively, are novel. There are previous reports with G23 mutated to D23 in melanoma kindreds.[24] However, there are no reports of the V28 being mutated in melanoma kindreds. A single base substitution, $g_{-33}\rightarrow c$ in the 5′-untranslated region of the *CDKN2A* gene was detected in one individual. This change has been observed in other individuals with melanoma, but the significance of this mutation is unknown currently.[24] The polymorphism, A148T, in exon 2 of *CDKN2A* gene was observed in nine individuals. Only one of these individuals had a family history of melanoma.

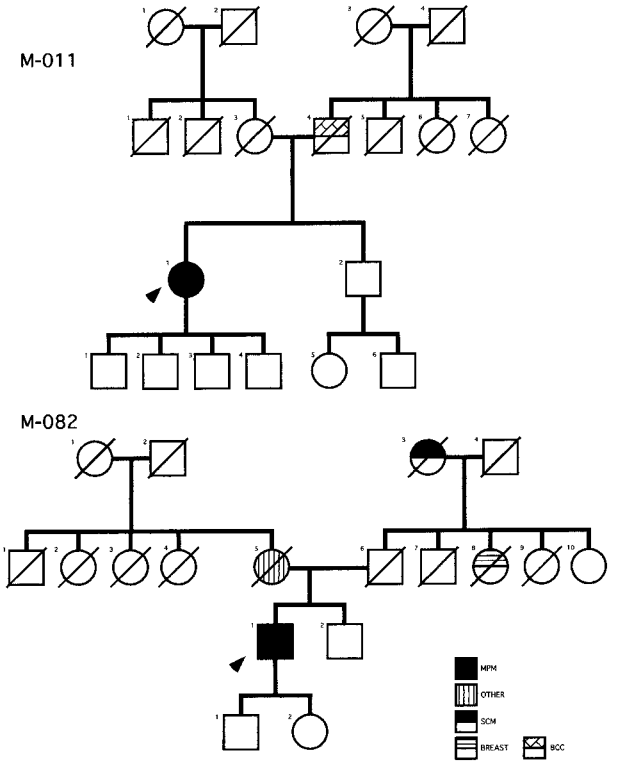

FIGURE 1. The family history of two individuals, M-011 and M-082, with mutation G101W in the coding sequence of CDKN2A gene. MPM: multiple primary melanoma; SCM: single cutaneous melanoma; BCC: basal cell carcinoma. Arrowheads indicate the proband.

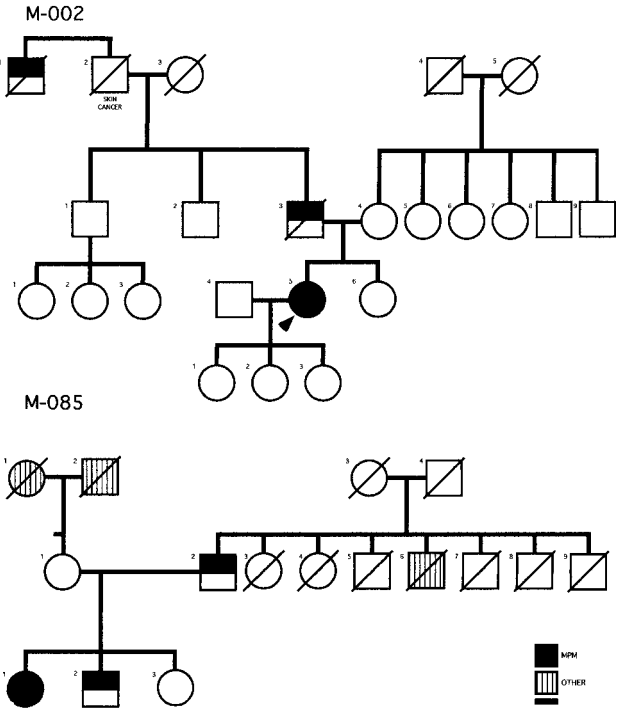

FIGURE 2. The family history of two individuals, M-002 and M-085, with mutation M53I in the coding sequence of CDKN2A gene. MPM: multiple primary melanoma; SCM: single cutaneous melanoma; BCC: basal cell carcinoma.

Pedigrees of Mutation Positive Families

The pedigrees of individuals with mutations are included in Figures 1, 2, and 3. Figure 1 shows pedigrees of families with the G101W mutation. This is one of the most common mutations seen worldwide.[22] Three American families with the G101W mutation have been reported previously.[25,26] These families had an average of 4 melanoma patients with a median age at diagnosis of 28 years. In contrast, the individuals with G101W mutation in our study showed limited family history. The proband from the first family, M-011, has had two primary melanomas with the first one diagnosed at age 64 years. No other member of this family was affected with melanoma, and the father of the proband had a BCC. The proband from the second family (M-082) had early onset melanoma at the age of 21 years followed by 5 additional primary malignant melanomas that were diagnosed at ages 33, 40, 44, and 45 years (when 2 were found). The paternal grandmother had a single cutaneous melanoma with unknown age of onset. The G101W families represent German, English, and Irish backgrounds.

The pedigrees in Figure 2 represent the families of two individuals with the M53I mutation. Both of these families had three members affected with melanoma. The previously characterized families were ascertained as melanoma kindreds with the number of affected members ranging from 2 to 15.[21]

Finally, the pedigrees included in Figure 3 represent the two families with frameshift mutations, 279del14 and 349del16, respectively. The family M-041 has seven members affected with melanoma. The proband, who had his first melanoma diagnosed at age 36 years, is the only member in this family with 3 primaries. In contrast, the proband from family M-086 has had two primary melanomas with no family history of melanoma. Breast carcinoma in the mother and a malignancy of unknown type in the paternal uncle are the only recorded cases of cancer in this family. The mutation 279del14 leads to a change in the reading frame with altered amino acid starting at amino acid 81 and a premature stop codon at amino acid 114. The other mutation, 349del16, translates to an altered sequence downstream from amino acid 104 and results in a premature stop codon at position 140. The protein product of *CDKN2A* gene harbors 4 ankyrin repeats that are protein motifs that are approximately 33 amino acids long and involved in protein–protein interactions.[27] Therefore, in family M-041, both the third and fourth ankyrin domains will be eliminated and

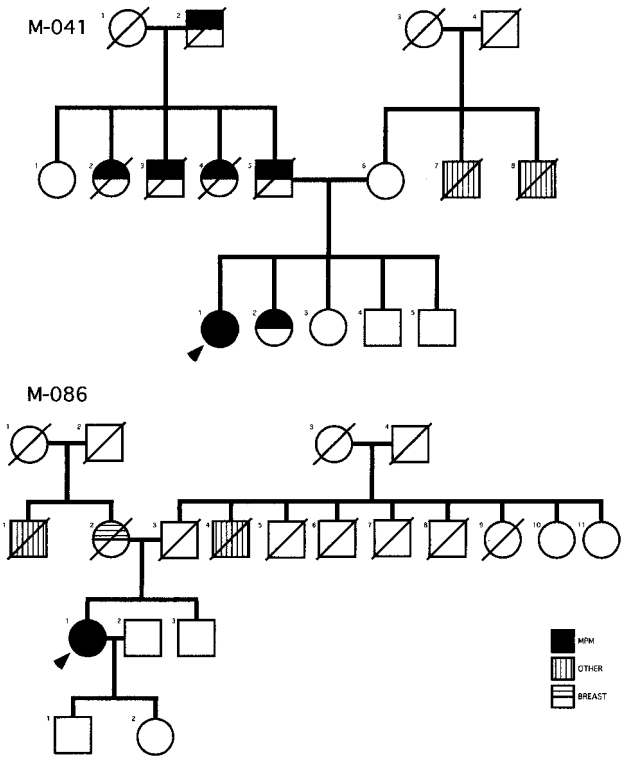

FIGURE 3. Two individuals were identified with frameshift mutations, 279del14 and 349del16. This figure shows the family history of melanoma and other cancer in the respective families. MPM: multiple primary melanoma; SCM: single cutaneous melanoma; BCC: basal cell carcinoma.

will lead to a highly unstable protein.[27,28] In family M-086, only the fourth ankyrin domain is altered. The phenotypic expression of the disease in these two families is very different (Fig. 3).

The presence of a germline mutation in individuals without a family history can suggest one of two things. Either the mutations observed are de novo mutations or the mutations are present in the families but there is variable expression. However, as can be seen from the pedigrees, many key individuals were deceased. Therefore, the origin of the germline mutations could not be determined. Environmental exposure is another explanation for variable phenotype and is equally plausible. Often, misclassification of the family history with respect to the clinical status of "unaffected" individuals also can be misleading.

DISCUSSION

There were three primary objectives of this study. The first was to estimate the likelihood of a family history of melanoma in individuals diagnosed with MPM. The second was to describe the clinical features of MPM cases. The third was to assess the association of germline mutations in the *CDKN2A* gene with MPM.

TABLE 5
Germline *CDKN2A* Mutation and Familial Risk Factors

Risk factor family history	Mutation negative (%)	Mutation positive (%)	*P* value
Melanoma	29/77 (37.7)	6/9 (66.7)	0.10
DN	10/40 (25.0)	1/4 (25.0)	1.00
Breast carcinoma	24/72 (33.3)	2/9 (22.2)	0.50

DN: dysplastic nevi.

With respect to the first objective, we found that 45% of individuals with MPM have a family history of melanoma (Table 1). This number is higher than in a previous report in which 24% cases of MPM had a family history.[29] In the current study, the definition of family history includes one or more affected family members who can be first-, second-, or third-degree relatives. In previous reports, the family history was considered positive only if at least one first-degree relative was affected with melanoma and was more restrictive.[29] At the same time, there is a significant proportion (51%) of MPM cases without any family history of melanoma.

With respect to the second objective of identifying potentially distinguishing clinical features, we found that individuals with MPM have a high prevalence of DN, lesions that are strong risk factors for melanoma (Table 2). The MPM cases commonly had a history of BCC as well. In addition, one recurrent feature among cases of MPM is the presence of at least one first- or second-degree relative with another common cancer. Basal cell carcinoma, squamous cell carcinoma, or breast carcinoma were the most frequent. Basal cell carcinoma is the most common skin carcinoma in the Western world and may reflect the high melanoma risk associated with heavy sun exposure.[30] Other reports also have indicated association of BCC with MPM.[20] There are previous reports of pancreatic carcinoma in individuals carrying the *CDKN2A* mutation.[17,31] However, we had only one case of pancreatic carcinoma in this series, and this was in a first-degree relative of a *CDKN2A* mutation negative melanoma case.

To address the third objective, we sought to find familial and personal risk factors in patients with MPM predictive of a germline mutation in the *CDKN2A* gene (Tables 5–7). However, because there were few mutation positive individuals in the current study, we cannot make strong statements about the association of *CDKN2A* mutations with putative familial risk factors. Not surprisingly, the data summarized in Table 5 suggest that a family history of melanoma is associated with a *CDKN2A* mutation. However, there

TABLE 6
Germline *CDKN2A* Mutations and Personal Risk Factors

Risk factor personal history	Mutation negative (%)	Mutation positive (%)	*P* value
BCC	38/87 (43.0)	0/9 (0)	0.02
DN	41/55 (73.9)	5/9 (60.0)	0.51

BCC: basal cell carcinoma; DN: dysplastic nevi.

TABLE 7
Germline *CDKN2A* Mutations and Age of Onset

Time factor	Mutation negative	Mutation positive	*P* value
Average age first melanoma (±SD)	50.0 (14.0)	39.7 (15.4)	0.09
Average years between melanomas (±SD)	4.2 (4.8)	2.0 (2.4)	0.28

SD: standard deviation.

is little to suggest a strong correlation with a family history of DN, BCC, or breast carcinoma.[29,32]

Candidate personal risk factors for a *CDKN2A* mutation included the presence of DN, a history of BCC, age of onset of the first melanoma, and the interval between primary melanomas. Although there was a high prevalence of DN in patients with MPM, this was true of those with and without mutations. Unexpectedly, a history of BCC characterized patients without a mutation (Table 6). The median age of diagnosis of the first melanoma in MPM cases was 47 years (Table 2). Table 7 shows the association of mutation status with age of diagnosis of the first melanoma and the time in years between melanomas. Although patients with mutations were younger and the intervals between their primaries were shorter, these differences were not statistically significant. Development of a risk model for predicting the mutational status of MPM patients will require a larger series of patients.

With respect to the novel mutations reported here, in the absence of functional assays for each mutation, only inferences can be made about their consequences. Nevertheless, each mutation is likely to be disruptive of function. Mutations are usually significant when they involve amino acids that have been conserved through evolution and/or reside in domains of known function. The novel mutations, G23C and V28G, involve amino acids conserved in species that diverged millions of years ago. The missense mutations G23C and V28G, as well as the truncating mutations, are expected to disrupt the ankyrin repeat domains and hence to compromise the function of the protein product.

CONCLUSIONS

Analysis of this series of MPM cases demonstrates the presence of germline mutations in *CDKN2A* in a small, but significant, fraction of such individuals (8.33%). Although no personal or familial trait of MPM cases emerged as a predictor of mutational status, their families had a substantial incidence of melanoma, DN, and BCC. These findings indicate that both MPM index cases and their family members should benefit from avoidance of excessive sun exposure and from a program of screening, self-examination, and regular professional surveillance for melanoma, DN, and BCC. Although screening for *CDKN2A* mutations in MPM cases and their family members is certainly not mandated, these individuals are very good targets for genetic studies to identify new, non-*CDKN2A* melanoma susceptibility genes.

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